The bacterial community associated with adult vine weevil (Otiorhynchus sulcatus) in UK populations growing on strawberry is dominated by Candidatus Nardonella

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Title

The bacterial community associated with adult vine weevil, *Otiorhynchus sulcatus* Fabricius, UK populations growing on strawberry (*Fragaria x ananassa*), is dominated by *Candidatus Nardonella*

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Short Title:

The Vine weevil bacterial microbiota

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Abstract

Otiorhynchus sulcatus Fabricius, commonly known as black vine weevil or simply vine weevil, is an important pest of soft fruit and ornamental crops. This species is endemic to temperate areas of Europe but has spread to many other areas over the last century, including North America and Australasia. The ability of vine weevils to adapt to such different environments is difficult to reconcile with the parthenogenetic reproduction strategy, which is likely to underpin a low genetic diversity. It is therefore tempting to hypothesize that weevil adaptation to different environments is mediated, at least partly, by the microbial communities inhabiting these insects. As a first step towards testing this hypothesis we characterised the composition of the bacterial microbiota in weevils from populations feeding on strawberry plants across four geographically-separate locations in the United Kingdom. We performed 16S rRNA gene Illumina amplicon sequencing, generating 2,882,853 high-quality reads. Ecological indices, namely Chao1 and Shannon, revealed that the populations used for this study harboured a low diversity and an uneven bacterial microbiota. Furthermore, β-diversity analysis failed to identify a clear association between microbiota composition and location. Notably, a single Operational Taxonomic Unit (OTU) phylogenetically related to *Candidatus Nardonella* accounted for 81% of the total sequencing reads for all tested insects. Our results indicate that vine weevil bacterial microbiota resembles other insects as it has low diversity and it is dominated by few taxa. A prediction of this observation is that location *per se* may not be a determinant of the microbiota inhabiting weevil populations. Rather, other or additional selective pressures, such as the plant species used as a food source, ultimately shape the weevil bacterial microbiota. Our results will serve as a reference framework to investigate other or additional hypotheses aimed at elucidating vine weevil adaptation to its environment.

Introduction

The association between insects and bacteria has received significant interest in recent decades as many studies have demonstrated the potential importance of these partnerships for insect fitness. Stable associations between two or more organisms, frequently termed symbiosis, is a widespread phenomenon in nature with outcomes ranging from negative to neutral to beneficial, often classified as parasitism, commensalism or mutualism, respectively. These associations can be categorized based on the grade of dependency as primary symbionts, which show strong interdependence and have typically long co-evolutionary history with the host, and facultative symbionts, which show more recent association and are not strongly interdependent. Research on insect-bacteria associations have often focused on pairwise mutualist symbiotic relationships from which insects acquire quantifiable benefits, although often the bacterial community harbored by insects is poorly characterized. Some insects with restricted diets rely on bacteria to compensate nutritional deficiencies. For instance, the pea aphid *Acyrthosiphon pisum* Harris is provided with essential amino acids and the vitamin riboflavin by its obligate endosymbiotic bacterium *Buchnera aphidicola* (Nakabachi & Ishikawa, 1999) and the tsetse fly *Glossina morsitans* Westwood is provided with essential vitamins by the endosymbiotic bacterium *Wigglesworthia glossinidia* (Nogge, 1981). Furthermore, bacteria can improve insect host fitness by degrading toxic secondary metabolites produced by plants as a chemical defense. This is the case for the coffee berry borer *Hypothenemus hampei* Ferrari which harbors *Pseudomonas* bacteria that detoxify caffeine by expressing caffeine demethylase genes (Ceja-Navarro et al., 2015). Importantly, certain bacteria have been shown to render their insect hosts less susceptible to predators and pathogens. This has been illustrated for the pea aphid, which is protected from parasitism by the parasitoid wasp *Aphidus ervi* Haliday when aphids are infected with the bacterium *Hamiltonella defensa* (Oliver

et al., 2005; Oliver et al., 2003) and from infection by the entomopathogenic fungus *Pandora neoaphidis* Remaud & Hennebert when aphids harbor the bacterium *Regiella insecticola* (Scarborough et al., 2005), and for the fruit fly *Drosophila melanogaster* Meigen, which becomes more resistant to RNA viruses when infected with the bacterium *Wolbachia* (Hedges et al., 2008). Weevils belong to the superfamily Curculionoidea which is one of the largest insect groups with more than 60,000 described species (Lyal & Alonso-Zarazaga, 2006). Weevil-associated bacteria studies, similarly to research on other insects, have typically focused on the symbiotic association between the bacterium *Nardonella* and different weevil species. Research started at the beginning of the 1990s with the observation of intracellular microorganisms confined in specialized cells, called bacteriocytes, in the rice weevil *Calandra oryzae* Linnaeus, although it remained undetermined whether the observed bacteria constituted a "symbiotic organ" or were simply "accessory cells" (Mansour, 1927; 1930; Pierantoni, 1927). Further investigation combining molecular techniques and fitness measures showed that these bacteria were present in different weevil species and were involved in adult development (Campbell et al., 1992; Nardon & Grenier, 1988). Nonetheless, it was not until the beginning of the 21st century that Lefevre et al. (2004), based on a phylogenetic analysis of the 16S rRNA gene, identified this microorganism as a γ-proteobacterium and designated the new lineage *Candidatus Nardonella*. This bacterium has been shown to be widespread throughout the weevil superfamily and is estimated to have become associated with weevils 125 million years ago (Conord et al., 2008; Lefevre et al., 2004). Nevertheless, some studies revealed that *Nardonella* has been replaced by another bacterium in species of the genus *Curculio* and the tribe Curculionini, highlighting the dynamic nature of insect-bacteria associations (Toju et al., 2010; Toju et al., 2013). Subsequent studies focused on identifying *C. Nardonella* in other weevil species and on studying other features of its biology,

~~such as population dynamics during different insect life stages or the location of the *Nardonella* bacteriocytes in insect tissues (Conord et al., 2008; Hosokawa & Fukatsu, 2010; Hosokawa et al., 2015; Huang et al., 2016; Mansour, 1930; Nardon et al., 2002; Toju & Fukatsu, 2011). Importantly, Anbutsu et al. (2017) working on the black hard weevil *Pachyrhynchus infernalis* Fairmaire showed that *Nardonella* is involved in cuticle formation by contributing to tyrosine synthesis as its suppression produced adults with low tyrosine titers and reddish, crumpled and/or deformed elytra.~~

Vine weevils, *Otiorhynchus sulcatus*, are parthenogenetic triploid females endemic to central Europe (Moorhouse et al., 1992). In the last two centuries, vine weevil distribution has expanded rapidly, primarily through plant trade routes, and this species is now found in most parts of Europe, and in parts of North America, South America, New Zealand and Japan (Kingsley, 1898; Masaki et al., 1984; Moorhouse et al., 1992; Prado, 1988). Vine weevils have been recorded developing successfully on 150 different host plant species (Moorhouse et al., 1992; Smith, 1932; Warner & Negley, 1976) with particular preference for strawberry (Hanula, 1988; van Tol et al., 2004; van Tol & Visser, 1998). Based on the ability of vine weevil to invade and establish in different environments despite its parthenogenetic reproduction mode, we hypothesized that the bacterial community associated with vine weevils could play an important role in insect adaptation.

In the last decade, advances in sequencing and computational approaches have enabled the characterization of the microbial communities associated with both plant and animal eukaryotic hosts, i.e. their microbiotas, at an unprecedented depth (Hacquard et al., 2015). Perhaps not surprisingly, such advances have been exploited to gain novel insights into the ecology of weevil microbiota. For instance, Hirsch et al. (2012) revealed that parthenogenetic species tend to harbor a less diverse bacterial community in comparison with sexual species in the weevil genus

Otiorhynchus. White et al. (2015) studied the bacterial community associated with exotic and endemic weevils in New Zealand and speculated that the presence of *Wolbachia* and *Rickettsia* could be involved in weevil resistance to parasitoids used in biocontrol. The influence of insect diet on shaping the bacterial microbiota composition was reported in the red palm weevil *Rhynchophorus ferrugineus* Olivier, the cotton boll weevil *Anthonomus grandis* Boheman and the pine weevil *Hylobius abietis* Linnaeus (Ben Guerrero et al., 2016; Berasategui et al., 2017; Montagna et al., 2015). Research by Berasategui et al. (2016) on the bacterial community composition in pine weevil populations across Europe revealed that despite significant variation in bacterial community composition, a core bacterial microbiota seemed to be shared by all pine weevil populations.

Many studies have shown that location can affect the bacterial microbiome of insects. For example, bacterial community richness and composition varied significantly between *D. melanogaster* populations collected from geographically separated areas of the USA (Corby-Harris et al., 2007). Furthermore, collection area was shown to clearly influence bacterial community assemblage of melon aphid, *Aphis gossypii* Glover, populations sampled across four Hawaiian Islands (Jones et al., 2011). Thus, as a first step to understand the influence of bacteria on vine weevil biology and fitness, we applied high-throughput sequencing techniques to investigate the existence of bacterial community patterns associated with location. For this purpose, we characterized the bacterial community associated with vine weevil populations infesting strawberry plants from geographically separated regions of the UK. Nevertheless, our results indicated that the sampled populations had a highly ~~conserved~~ similar bacterial community dominated by a single bacterial ~~sequence~~ phylotype, classified as *C. Nardonella*, which accounted for 81% of sequencing reads retrieved from all studied insects.

Materials and methods

Vine weevil adult populations

Vine weevil adults were collected during summer 2015, 2016 and 2017 from an area of approximately 50 m^2 within strawberry crops at five different sites across the UK. Insects collected at different locations were considered as different populations. Exceptionally, we considered insects collected at the Invergowrie site as two separated populations, despite coming from the same area, as they were collected in two consecutive years and could harbour different bacterial community influenced by the different environmental conditions experienced. Details of the collection sites are presented in Table 1 and Figure 1. The collection sites in Stafford were only separated by 766 m whereas the Shifnal and Woore collection sites were separated from these two sites an average distance of 30 km. The collection site in Invergowrie was 494 km distant in average from the rest of the sites. Following collection, insects were directly frozen with liquid N_2 and stored at -80°C until further use.

DNA extraction

DNA extraction was performed on eight insects from each population except for the Stafford_2 population in which four insects were used due to the small sample size at this site (one insect = one replicate). Insects were surface sterilised in a 1% bleach (May and Baker LTD, Dagenham, England) solution for one minute (Lawrence et al., 2015; Malacrinò et al., 2018). To remove the remaining bleach insects were submerged in autoclaved water three times, each time the insects were submerged for one minute. Surface sterilised insects were ground individually using pestle and mortar sterilised by exposing to UV light for 10 minutes. Once the whole sample was ground to a powder, total DNA was extracted using the NucleoSpin Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions and the alternative step suggested in the Kit

protocol. An additional incubation at 70°C for 10 minutes was included, after the 10 minutes lysis step at 65°C specified in the protocol, to lyse gram negative bacterial cell walls. Extracted DNA was stored at -20°C in autoclaved Eppendorf tubes until further use.

PCR amplification of the 16S rRNA gene

A fragment of the V4 hypervariable region of the 16S rRNA gene was used for the current bacterial community study as it has been shown to yield optimal community analysis in previous studies (Caporaso et al., 2011) and it was chosen as a reference marker for the Earth Microbiome Project (EMP) (Gilbert et al., 2010). The primers used, 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), carry an Illumina adapter, pad and linker at the 5' terminus. Additionally, the reverse primer (806R) carries a unique barcode which is a 12-base error correcting Golay code to allow multiplexing, i.e. sequencing different samples simultaneously.

The Kapa HiFi HotStart PCR kit (Kapa Biosystems, Wilmington, USA) was used to amplify the targeted DNA fragment in a G-Storm GS1 Thermal Cycler (Gene Technologies, Somerton, UK). The PCR mixture (20 µL) consisted of 4 µL of 5X Kapa HiFi Buffer, 1 µL of a 10 ng/µL Bovine Serum Albumin solution (Roche, Mannheim, Germany), 0.6 µL of a 10 mM Kapa dNTPs solution, 0.6 µL of a 10 µM solution of each primer, 0.25 µL of Kapa HiFi polymerase (0.02 U/µL), 8 µL of sterile water and 1 µL of a 10 ng/µL solution of the template DNA. Samples in the thermocycler were subjected to three minutes of DNA initial denaturation at 94°C, then 35 cycles of 30 seconds of DNA denaturation at 98°C, 30 seconds of primer annealing at 50°C, and one minute of DNA elongation at 72°C, followed by a final elongation step of 10 minutes at 72°C.

Based on the protocol described by Costello et al. (2009) and adopted by the EMP, each insect replicate was PCR amplified using a specific combination of forward and reverse primers with a

unique, replicate-specific, barcode. For each primer pair combination, the corresponding PCR reaction was performed in simultaneous triplicates to diminish amplification biases, with an additional no template control. PCR reactions were combined in a barcode-wise manner, i.e. amplification replicates of the same primer pair were mixed and were tested on a 1.5% agarose gel with the corresponding no template control. The simultaneous triplicate amplification procedure was repeated three times for each primer pair combination. So, for each primer pair combination we performed nine amplifications in total. Finally, all PCR products were mixed in a barcode-wise manner (nine amplifications mixed) and kept at -20°C until further use.

Illumina MiSeq library preparation and sequencing

PCR products were purified with Agencourt AMPure XP kit (Beckman Coulter, Brea, USA) using 0.7 µL AMPure XP beads per 1 µL of sample. The DNA concentration of 3 µL of each PCR reaction, mixed according to their barcode, was quantified using Picogreen (ThermoFisher, UK) following the manufacturer's recommendations. Next, the amplicon library was generated by mixing individual barcoded replicates in an equimolar ratio. The library was sequenced by the Genome technology group at the James Hutton Institute, Dundee UK, using Illumina MiSeq platform with paired-end reads of 150 bp per read.

Illumina MiSeq data processing with QIIME

The Illumina MiSeq platform generated three FASTQ files with the forward, reverse and barcode sequences. The FASTQ files and the metadata information, organised in a mapping file, were processed with the open source software Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.0 (Caporaso et al., 2010) using the default parameters unless otherwise specified.

Forward and reverse FASTQ files were decompressed and merged specifying a minimum sequence overlap of 5 bp between pairs of reads using the command 'join_paired_ends.py' The

reads were quality filtered and demultiplexed with the command 'split_libraries_fastq.py' specifying a minimum Phred quality score of 20. The remaining high-quality reads were clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity using SortMeRNA and sumaclust algorithms. OTUs were defined using a subsampled open-reference OTU picking approach with the command 'pick_open_reference_otus.py' against the chimera checked Greengenes database version 13_5 (DeSantis et al., 2006). The output was an OTU table with the identified OTUs as rows and the samples as columns, containing the abundance of each OTU per sample. The OTUs that did not match by 97% similarity any bacterial sequence on the database were classified as Unassigned.

Identification of the Unassigned OTU_0

The proportion of different Unassigned OTUs revealed that the dominant OTU was the OTU_0, which accounted for 99% (2,347,616 reads) of the total reads for Unassigned OTUs (2,364,356 reads). This OTU matched bacterial sequences found in different members of the Curculionidae family on the NCBI database. The highest matching percentage revealed similarity with bacterial sequences found in *Otiorhynchus sulcatus* Fabricius (vine weevil) by 100% (GenBank: Accession No. JN563788.1 and JN563787.1) and in *O. salicicola* Heyden (GenBank: Accession No. JN394467.1), *O. armadillo* Rossi (GenBank: Accession No. JN394466.1) and *O. rugostriatus* Goeze (GenBank: Accession No. JN394465.1) by 98% (Hirsch et al., 2012). Furthermore, it matched bacterial sequences found in *Listronotus bonariensis* Kuschel by 96% (GenBank: Accession No. KJ522448.1) (White et al., 2015), in *Steriphus variabilis* Broun by 93% (GenBank: Accession No. KJ522449.1) (White et al., 2015) and a bacterial sequence classified as *Candidatus Nardonella* (γ-proteobacteria) found in *Pachyrhynchus infernalis* by 92% (GenBank: Accession

No. AP018160.1) (Anbutsu et al., 2017). Hence, we have provisionally classified the OTU_0 as *C. Nardonella*.

Data analysis with R

To analyse the data with R software version 3.3.3 the packages phyloseq version 1.19.1 (McMurdie & Holmes, 2013) and PMCMR version 4.3 were installed from Bioconductor using the code 'source ("http://bioconductor.org/biocLite.R")' and the function 'biocLite()'. The packages dendextend version 1.8.0, vegan version 2.4-5, ape version 5.0 and ggplot2 version 3.0.0 were installed with the function 'install.packages'. The function ancom was installed using the code 'source("ancom_functions.R")' and 'source("plot_ancom.R")'.

First, a new OTU table was generated after filtering the initial OTU table obtained with QIIME ~~using the function 'prune' to remove~~ for OTUs classified as mitochondria or chloroplast, likely representing a contamination from host tissues and/or the food source. Next, we removed from the remaining OTUs list, instances matching OTUs identified as environmental contaminants of the laboratory where we generated our sequencing library (Pietrangelo et al., 2018) ~~likely representing insect and plant contamination,~~ . After this initial filtering *in silico*, we identified the most abundant OTU in the phylum Bacteroidetes was used as an outgroup to root the phylogenetic tree generated by QIIME. Third, the phyloseq package was used to create the phyloseq object combining the new OTU table, the taxonomy matrix, the phylogenetic tree and the mapping file using the command 'merge_phyloseq'. Fourth, the dataset was filtered to discard OTUs with less than five reads in at least ~~one of the populations~~ 10% of the studied insects with the function 'filter_taxa'.

To study the α-diversity, replicates were rarefied (Gotelli & Chao, 2013; Gotelli & Colwell, 2001; 2011) to a similar sequencing depth of 11,207 reads with the function 'rarefy_even_depth' from the package phyloseq. The Chao1 and Shannon indices were then calculated with the function

'estimate_richness' from the package phyloseq. Normality was tested by applying a Shapiro-Wilk test with the function 'shapiro.test' which revealed that only Shannon index values were not normally distributed. Therefore, data for Observed OTUs and Chao1 index were analysed with the parametric ANOVA test paired with Tukey test for multiple comparisons with the functions 'aov' and 'TukeyHSD' from the R stats package 3.3.3. Shannon index values were analysed with the non-parametric Kruskal-Wallis test using the functions 'Kruskal.test' and 'posthoc.kruskal.dunn.test' from the package PMCMR.

To study the β-diversity, the dataset was transformed into relative abundances, i.e. sample reads/total amount of reads. A distance matrix was calculated using Bray-Curtis metrics, which considers OTU relative abundance, with the function 'ordinate' from the package phyloseq. A hierarchical cluster analysis was performed with the function 'hclust' and the generated Cluster dendrogram was modified with the function 'set' within the package dendextend before plotting. Statistical differences in microbial composition among populations were tested using a permutational multivariate analysis of variance with the function 'adonis' from the package vegan (Dixon, 2003). OTUs showing significant differences in abundance between populations were revealed by applying an analysis of composition of microbiomes with the function 'ANCOM' from the package ANCOM using the multiple correction option '1'(Weiss et al., 2017).

Results

Vine weevil bacterial microbiota is composed of 85 different bacterial taxa

We characterized the bacterial community of six vine weevil populations collected from strawberry crops grown at different locations in the UK (Table 1 and Figure 1) using an Illumina MiSeq 16S rRNA gene sequencing approach. The sequencing library yielded 3,153,991 high-quality reads which clustered in 994 Operational Taxonomic Units (OTUs) at 97% similarity.

OTUs classified as chloroplast and mitochondria, as well as predicted contaminant OTUs, were removed from the original file, which reduced the number of high-quality reads to 2,882,853 (per sample mean 65,519; max 199,121; and min 11,224) and the number of OTUs to 931. As a result, 91% and 93% of the original reads and OTUs, respectively, were kept for further analysis. To discard low abundance OTUs, which have low reproducibility, ~~only those~~ OTUs that had less ~~more~~ than five reads in at least 10% of the studied insects were removed ~~retained~~ for subsequent analysis. This further reduced the number of reads to 2,871,373 and the number of OTUs to 85. Although this step reduced the number of OTUs by over 90%, we retained more than 99% of the total number of high-quality reads. This suggested that the bacterial microbiota of the populations tested in this study comprised a relatively low number of highly abundant bacterial taxa.

Vine weevil bacterial microbiota is dominated by γ-proteobacteria and α-proteobacteria

To investigate the taxonomic distribution at genus level, we manually annotated the OTU_0 as *C. Nardonella* and imposed a threshold of 1% abundance on the whole dataset for plotting purposes.~~We investigated the taxonomic distribution, focusing on bacterial genera classes with a relative abundance greater than 1% on the whole dataset~~. As a result, only two bacterial genera ~~classes~~ and one family, that could not be classified at genus level, were considered: *Candidatus Nardonella* (γ-proteobacteria) and *Rickettsia* and Rickettsiaceae (α-proteobacteria) with average relative abundance of 85%, 5.8% and 6.9%, respectively (Figure 2). ~~These two bacterial genus classes and family, accounted for 97.7% of the total reads generated for each of the studied insects across the 6 vine weevil populations.~~ This further supports the idea that vine weevil bacterial microbiota in the sampled insects was dominated by a small number of taxa.

Vine weevil populations harbor a low diversity bacterial microbiota

Within population diversity, or α-diversity, computed at OTU level, revealed low diversity in the bacterial communities across vine weevil populations. On average, populations harbored a bacterial community comprising 36 OTUs, a richness value (Chao1 index) of 43 and an evenness value (Shannon index) of 0.5 (Figure 3). ~~Invergowrie populations tended to harbor a less diverse and more uneven bacterial community compared to the other populations.~~ Statistical analysis of the observed OTUs revealed that Invergowrie populations tended to harbor a lower number of OTUs (Figure 3A, ANOVA, F =20.16, df= 5, P< 0.05) and lower richness index values (Figure 3B, ANOVA, F= 16.89, df=5, P< 0.05) compared to the rest of the populations, although Stafford_2 and Invergowrie_2 populations were not significantly different ~~(Figure 2A, ANOVA, H=34.13, df=5, P< 0.05)~~. ~~Statistical analysis of richness values revealed the existence of three groups with high (Stafford_1 and Woore populations), intermediate (Stafford_2 and Shifnal populations) and low (Invergowrie_1 and Invergowrie_2 populations) diversity~~ ~~(Figure 2B, Kruskal-Wallis test, H= 25.28, df=5, P< 0.05).~~ ~~However,~~ . Statistical analysis of Shannon index values revealed that evenness was significantly lower only for Stafford_2 and Invergowrie_1 populations, compared to the rest of the populations (Figure 3C, Kruskal-Wallis test, H= 19.88, df=5, P< 0.05).

Vine weevil bacterial microbiota composition is dominated by *Candidatus Nardonella*.

Vine weevil bacterial community diversity between populations, or β-diversity, was calculated using a Bray Curtis approach, which considers OTU relative abundance. This analysis failed to reveal a clear pattern associated with location ~~as the maximum level of variation between samples was only 30%~~ (Figure 4). ~~Nevertheless, statistical analysis revealed that despite the high similarity between samples, there were significant differences in the bacterial community composition between populations (Adonis test, df=5, P<0.05).~~ ~~We performed a rank-abundance evaluation of~~

Closer inspection of the individual OTUs identified in our library ~~to detect the microbiological basis underpinning the apparent lack of variation in OTU composition across sites. This analysis~~ revealed that samples were dominated by the OTU_0, classified as *C. Nardonella*, which represented 81% of the total sequencing reads and 84%, on average, of the sequencing reads assigned to each individual insect ~~(Figure 4)~~. Thus, the high incidence of a single bacterial phylotype classified as *C. Nardonella* governed the bacterial community assembly of the populations studied here.

Location specific OTUs are dominated by members of the Proteobacteria phylum

Statistical analysis revealed that despite the lack of location-associated pattern in the microbiota composition, ~~the high similarity in bacterial community composition~~, ~~there~~ we identified ~~were~~ significant differences between populations (Adonis test, df=5, $P<0.05$, R2 Location= 0.37). ~~We further investigated the presence of significantly different OTUs among populations.~~ A total number of 16 OTUs was shown to vary significantly in abundance between vine weevil populations with 11, 2 and 1 of the OTUs belonging to Proteobacteria, Bacteroidetes and Actinobacteria phyla, respectively, and 2 Unassigned OTUs (ANCOM test, $P<0.01$, multiple test correction). OTUs assigned to Proteobacteria phylum belonged to Sphingomonadales and Rickettsiales orders within α-proteobacteria and to Enterobacteriales, Pseudomonadales and Xanthomonadales orders within γ-proteobacteria. OTUs assigned to Bacteroidetes phylum belonged to Sphingobacteriales and Flavobacteriales orders, and OTUs assigned to Actinobacteria phylum belonged to Actinomycetales order. The average abundance for these OTUs per population was: 0.05% for Stafford_1, 0.02% for Stafford_2, 0.08% for Shifnal, 0.12% for Woore, 0.02% for Invergowrie_1 and 0.02% for Invergowrie_2. Thus, OTUs that varied in abundance between locations represented a small fraction of the total number of reads and, despite belonging to

different phyla, they were biased towards members of the Proteobacteria phylum. This observation suggests that the 37% of the variance attributed to location in the analysis, is associated, at least partially, to the fluctuation of *C. Nardonella* across populations.

Discussion

The current study characterized for the first time the bacterial community of vine weevil adults from five different UK geographic areas. Our results showed that the bacterial microbiota composition did not follow a pattern governed by location, as only a small fraction of the Operational Taxonomic Units (OTUs) varied in abundance between populations. Furthermore, the bacterial community was dominated by members of the Proteobacteria phylum, with remarkably high abundance of a single bacterium belonging to the γ-proteobacteria and classified as *Candidatus Nardonella.* These findings are consistent with those reported previously in insect bacterial community studies, which revealed a similarly low diversity of bacterial microbiota dominated by members of the Proteobacteria phylum, compared with analogous studies on vertebrates or soil (Bansal et al., 2014; Bili et al., 2016; Broderick et al., 2004; Chandler et al., 2011; Colman et al., 2012; Corby-Harris et al., 2007; Douglas, 2011; Fierer & Jackson, 2006; Gauthier et al., 2015; Ishak et al., 2011; Jones et al., 2013; Robertson-Albertyn et al., 2017; Vasanthakumar et al., 2006; Wong et al., 2011; Yun et al., 2014). This bacterial microbiota pattern seems to be common across insect clades even when targeting different 16S rRNA gene hypervariable regions (Baker et al., 2003; Guo et al., 2013; Suzuki & Giovannoni, 1996; Yang et al., 2016) or applying different DNA extraction procedures (Martin-Laurent et al., 2001). The reasons underlying such an intriguing pattern remain undetermined, although a number of hypotheses have been proposed to explain low microbial diversity in insects. One hypothesis suggests that the insect immune system fine tunes the bacterial microbiota composition in order to

tolerate only beneficial bacteria as has been seen in *D. melanogaster* and the red palm weevil (Chandler et al., 2011; Dawadi et al., 2018; Lhocine et al., 2008; Login et al., 2011; Ryu et al., 2008). Another hypothesis, although not exclusive, suggests that low microbial diversity results from negative interactions between co-inhabiting bacteria as has been seen between *Buchnera* and *Rickettsia* in the pea aphid (Sakurai et al., 2005), between *Spiroplasma* and *Wolbachia* in *D. melanogaster* (Goto et al., 2006) and between *Bartonella* and *Rickettsia* in fleas from the genus *Oropsylla* (Jones et al., 2012). Nonetheless, the biological factors shaping insect bacterial microbiota in this characteristic manner remain speculative and open to future investigation.

The findings presented here show that vine weevil bacterial community is mainly composed of members of the α and γ-proteobacteria classes with noteworthy high abundance of the OTU classified as *C. Nardonella*. Conversely, a previous sequencing attempt to characterize vine weevil bacterial microbiota showed that it was composed entirely of members of the α-proteobacteria order and, surprisingly, *C. Nardonella* abundance was very low as it could only be detected by diagnostic PCR with specific primers (Hirsch et al., 2012). Differences between the previous and the current vine weevil bacterial microbiota characterization could be attributed to insect ontogeny as Hirsch et al. (2012) examined 24-72h old vine weevil larvae, whereas we used vine weevil adults close to maturity. Insect life stage has been shown to influence microbial community composition in several insects, for example the Hessian fly *Mayetiola destructor* Say (Bansal et al., 2014), species of the parasitoid wasp genus *Nasonia* (Brucker & Bordenstein, 2012), the rice water weevil *Lissorhoptrus oryzophilus* Kuschel (Huang et al., 2016), the southern pine beetle *Dendroctonus frontalis* Zimmermann (Vasanthakumar et al., 2006), the house fly *Musca domestica* Linnaeus (Wei et al., 2013), *D. melanogaster* (Wong et al., 2011) and the neotropical butterfly *Heliconius erato* Linnaeus (Hammer et al., 2014). Furthermore, *Nardonella* in rice water

weevil was present at low titer in larvae and pupae whereas its abundance increased substantially upon adult emergence (Huang et al., 2016). The mechanisms triggering such developmental changes in microbial composition are unclear, although it has been proposed that adaptation to utilize different resources at different life stages could influence bacterial community composition (Hammer et al., 2014). An additional factor to consider is that Hirsch et al. (2012) used larvae hatched from surface sterilized eggs for bacterial community characterization. Although bacterial transmission to progeny through the egg surface has not been studied in vine weevil, egg surface sterilization could potentially eliminate an important source of bacteria for the developing insect as has been described in other members of the Coleoptera order, such as the reed beetle genus *Macroplea* (Kleinschmidt & Kölsch, 2011; Kölsch et al., 2009) and the rove beetle *Paederus sabaeus* Erichson (Kellner, 2001; 2002). Therefore, to clarify the differences between the two studies, further research should aim to characterize vine weevil larvae bacterial microbiota in comparison with egg and adult life stages.

~~Interestingly, the vine weevil populations considered in our study harbored highly conserved bacterial communities despite belonging to geographically separate areas. This could indicate that vine weevil diet plays a major role in shaping bacterial community composition, as all individuals were collected from the same host plant species. Insect diet has been proposed as an important factor influencing bacterial community composition for many insect species (Broderick et al., 2004; Chandler et al., 2011; Colman et al., 2012; Violetta et al., 2017; Yun et al., 2014). Furthermore, diet influence on bacterial community composition has been acknowledged in closely related members of the weevil superfamily Curculionoidea: the red palm weevil experienced a dramatic change in bacterial community composition after 30 days of feeding on apple, compared with the original population from which these insects were sampled (Montagna~~

et al., 2015); the pine weevil possesses a bacterial microbiota composition resembling that of other bark beetles exploiting the same food source, whereas it differs from closely related weevils exploiting different food sources (Berasategui et al., 2016); populations of the chestnut weevil *Curculio sikkimensis* Hell collected from different *Quercus* species harbored different bacterial microbiota (Toju & Fukatsu, 2011); and the bacterial community of cotton boll weevil *Anthonomus grandis* Boheman changed significantly when fed with different artificial diets (Ben Guerrero et al., 2016). Thus, to confirm that diet is a dominant factor affecting microbial composition in vine weevils, future research should consider characterizing the bacterial community of populations from the same location infesting different host plant species.

Perhaps unexpectedly, location specific bacteria detected in our study constituted a small fraction of the total number of reads suggesting that location has a limited role in sculpting the composition of vine weevil bacterial microbiota. However, caution should be exerted when interpreting these data. For instance, our study could be limited by considering a relatively narrow sampling area. Furthermore, Shifnal and Woore populations lacked sampling replicates as we only analyzed one population at those locations. Hence, the greater proportion of location specific OTUs on Woore population, compared with the rest of the populations, may be derived from the sampling design rather than the intrinsic biology of the populations. Thus, future studies should aim to collect insects from a wider geographic area, including different populations from the same area, to determine if location has an influence in bacterial community composition in vine weevil.

The high incidence of the OTU classified as *C. Nardonella* in all tested insects could indicate the importance of its contribution to adult development and cuticle integrity as has been demonstrated in studies of other weevil species (Anbutsu et al., 2017; Kuriwada et al., 2010). *C. Nardonella* is a bacterial symbiont widespread throughout the weevil superfamily located in bacteriocytes

forming a specialized organ, the bacteriome, which localizes at the foregut/midgut junction of larvae and at the apex of the ovarioles in adults (Conord et al., 2008; Hosokawa & Fukatsu, 2010; Hosokawa et al., 2015; Huang et al., 2016; Mansour, 1930; Nardon et al., 2002). In a recent study, the *Nardonella* genome was sequenced from the black hard weevil *Pachyrhynchus infernalis* revealing that it possesses an extremely small genome (0.20 to 0.23 Mb) with reduced metabolic capacity (Anbutsu et al., 2017), a characteristic feature for primary obligate symbionts (Moya et al., 2008). Results from the same study revealed that this bacterium could influence adult development through its involvement in tyrosine production. Therefore, based on the contribution of *Nardonella* to adult development in other weevil species, it would be of great interest to investigate the dynamics of this bacterium at all vine weevil life stages.

The findings of the present study contribute to the field of research on insect bacterial microbiota as we have comprehensively characterized vine weevil bacterial community of several insect populations by amplifying a region of the V4 hypervariable region of the prokaryotic 16S rRNA gene, paired with Illumina MiSeq sequencing technology. Moreover, our results showed that vine weevil bacterial community of the populations sampled from strawberry plants did not follow a location specific pattern and was dominated by a single bacterium identified as *C. Nardonella*. This study forms the basis for future research to understand the role of ~~diet and other~~ location-specific ~~factors such as~~ biotic and abiotic factors ~~climatic conditions and natural enemy pressures~~ in shaping vine weevil bacterial community. An additional interesting line of research would be to study the importance of *C. Nardonella* for vine weevil development and or reproduction. Likewise, as innovations in sequencing technology are becoming available for experimentation, it will be interesting to accurately identify and quantify the dominance of *C. Nardonella* in the vine weevil microbiota with additional methodologies. This will provide valuable insights for the field of

agroecology to devise new strategies for management and biocontrol of this damaging and polyphagous insect pest.

Data Availability

The sequences generated in this study are deposited in the European Nucleotide Archive (ENA) under the study accession number PRJEB28361. The script used to analyze the data and generate the figures in this study is available on GitHub athttps://github.com/BulgarelliD-Lab/

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Figure legends

Figure 1. Location of vine weevil sampling areas across the UK. Each shape represents a population collection site.

Figure 2. Taxonomic classification of bacterial community members at genus ~~class~~ level. ~~α-proteobacteria (filled area) and γ-proteobacteria (unfilled area) are shown.~~ Y-axis represents average relative abundance in percentage of reads. Bars represent each insect from the ~~a~~ population specified on the x-axis. Populations are St1: Stafford_1, St2: Stafford_2, Shf: Shifnal, W: Woore, I1: Invergowrie_1 and I2: Invergowrie_2.

Figure 3. Observed OTUs, richness and evenness of bacterial communities. A) Average number of observed OTUs per population, B) average Chao1 index values of richness per population and C) average Shannon index values of evenness per population. Plotted values sharing the same letter were not significantly different.

Figure 4. Bray-Curtis cluster dendrogram based on dissimilarity of the bacterial community associated with each insect. Each dendrogram leaf represents a single insect and different shapes represent different populations.

Tables

Table 1. Vine weevil population location and year of collection.

POPULATION	LOCATION	YEAR
Stafford_1	Stafford, Staffordshire	2017
Stafford_2	Stafford, Staffordshire	2017
Shifnal	Shifnal, Shropshire	2015
Woore	Woore, Staffordshire	2015
Invergowrie_1	Invergowrie, Dundee	2017
Invergowrie_2	Invergowrie, Dundee	2016

Figure 1. Location of vine weevil sampling areas across the UK. Each shape represents a population collection site

933x724mm (72 x 72 DPI)

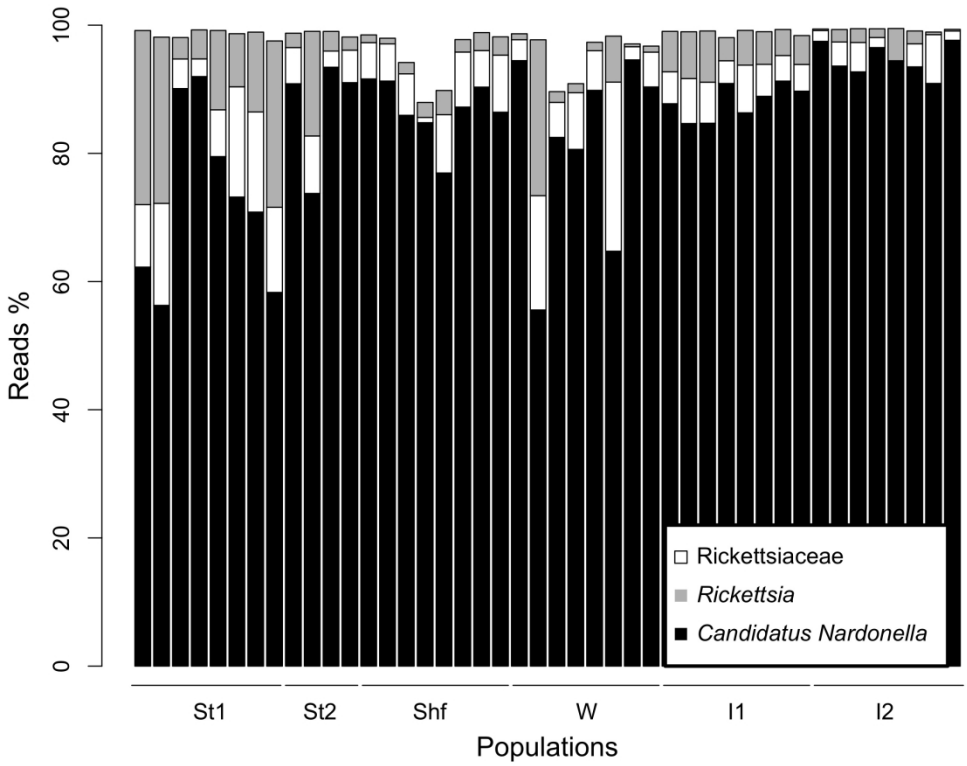

Figure 2. Taxonomic classification of bacterial community members at genus level. Y-axis represents average relative abundance in percentage of reads. Bars represent each insect from the population specified on the x-axis. Populations are St1: Stafford_1, St2: Stafford_2, Shf: Shifnal, W: Woore, I1: Invergowrie_1 and I2: Invergowrie_2.

933x724mm (72 x 72 DPI)

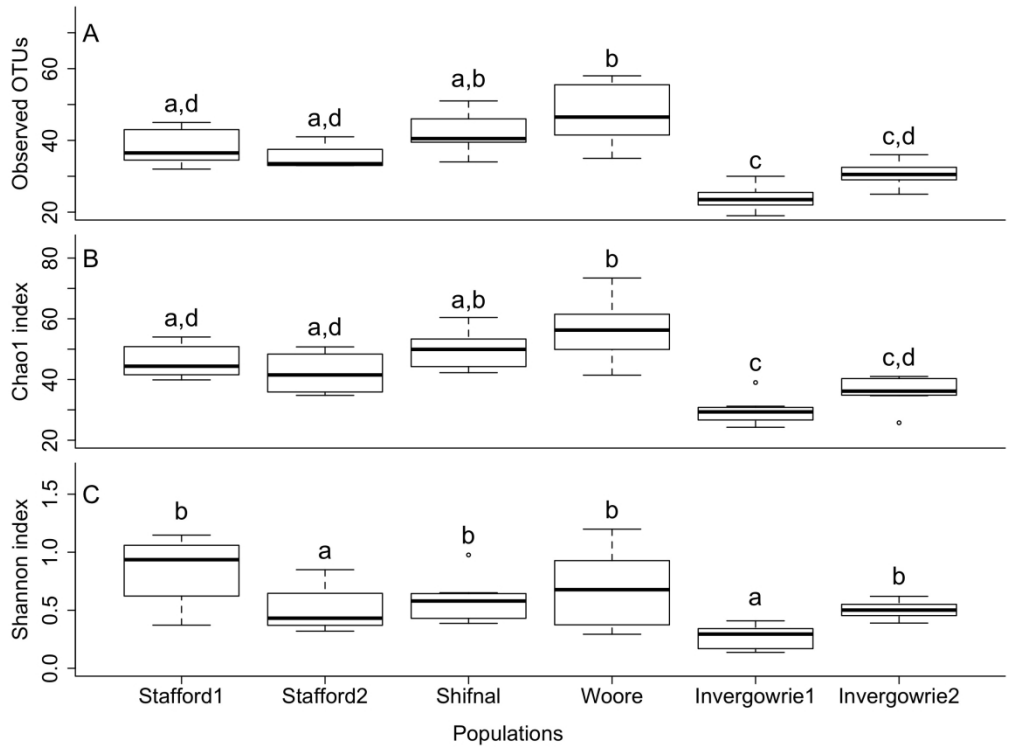

Figure 3. Observed OTUs, richness and evenness of bacterial communities. A) Average number of observed OTUs per population, B) average Chao1 index values of richness per population and C) average Shannon index values of evenness per population. Plotted values sharing the same letter were not significantly different.

933x724mm (72 x 72 DPI)

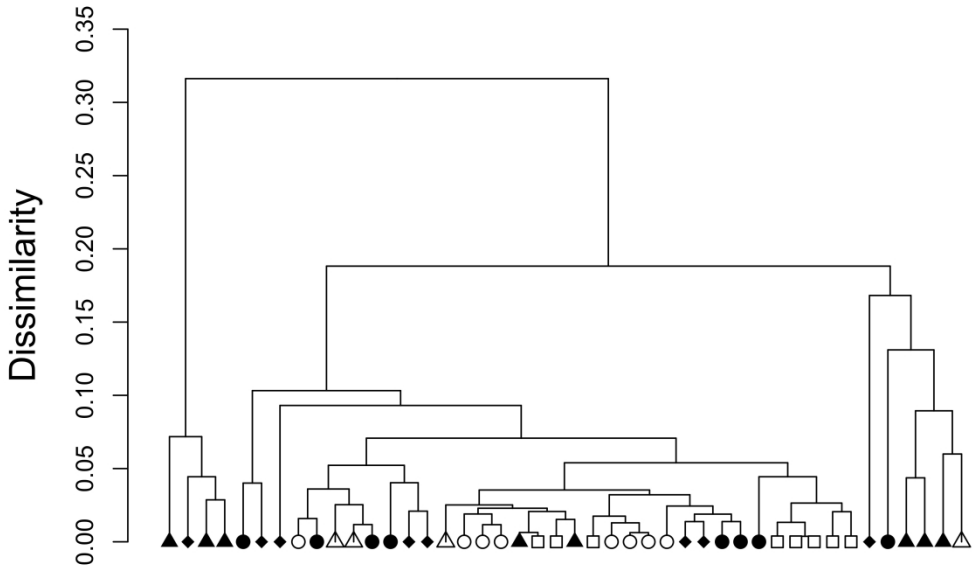

Figure 4. Bray-Curtis cluster dendrogram based on dissimilarity of the bacterial community associated with each insect. Each dendrogram leaf represents a single insect and different shapes represent different populations.

1458x833mm (72 x 72 DPI)